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IX. *A Conjugating "Yeast."*

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Communicated by Professor Marshall Ward, *F.R.S.*

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[Plate 46.]

The organism described in this paper was obtained from commercial ginger in a flask of saccharose-Mayer solution.* On keeping the flask at a temperature of 25° C., fermentation occurred, and a brownish-white deposit was formed. An examination of this deposit showed that it consisted chiefly of yeast-cells. Some of these cells were separated, and fractional series of six plate-cultures each were made. The plates were kept at the ordinary room temperature, and the colonies of yeast-cells were visible to the naked eye in three days. The colonies when a fortnight old appeared to the naked eye as small rounded white masses, about the size of a pin's head, with regular edges. Under the low power of the microscope the edges appeared fairly regular in those colonies which had developed on the surface of the gelatine, but the submerged colonies had a woolly appearance, due to numerous branches made up chiefly of yeast-cells placed end on end, and comparatively few side branches of such cell-systems, the branches radiating out from a central mass of yeast-cells. By means of hanging-drop cultures in beer-wort gelatine a single cell was selected under the microscope, and its development watched (see Plate 46, fig. 1), until the colony to which it gave rise was large enough to be visible to the naked eye. An infection was then taken from this colony, and further fractional series in beer-wort gelatine made; a streak-culture on beer-wort gelatine being made at the same time from the same infection. From the growths which developed on these streak- and plate-cultures, an abundant supply of the organism in a pure state was obtained.

The streak-culture was the method adopted for keeping a pure stock of the fungus.

The Growth on Plate-cultures.

Cultures made on plates of beer-wort gelatine show no special characteristic which distinguishes this form from many other yeasts. When observed with the naked

* Saccharine 15 grammes, Mayer's solution 100 cub. centims.

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eye, the colonies first become visible as small dots in 3–4 days, when the cultures are kept at the ordinary room temperature (15–20° C.). A week later they have reached the size of a pin's head, and appear as small, round, brownish-white milky looking masses, if growing on the surface of the gelatine. Such colonies viewed under the low power of the microscope have usually regular edges and are composed of a solid mass of yeast-cells. Submerged colonies present a different appearance, being more or less like woolly balls when viewed with a simple lens, and of a brownish colour, due no doubt to the colour of the gelatine in which they are imbedded. Their woolly appearance is seen under a low power to be produced by numerous branches radiating from a central mass of yeast-cells. These branches are made up of yeast-cells which have developed principally in an end-on-end fashion, lateral buddings being scanty.

The growth of the colonies is soon arrested, a larger size than that of a pin's head being rarely attained. In young colonies the cells are identical in appearance with those of an actively budding yeast. They are usually oval in shape, one or both ends often being more or less pointed; their contents appear homogeneous, the clear protoplasm filling the whole of the cell, except in cases where one or more small vacuoles similar in nature to those of an actively budding yeast are present; and the cell wall is not sharply marked off from the rest of the cell. Such cells produce buds actively in a typical yeast-like manner. (Plate 46, fig. 1.) As the colony grows older, the active budding ceases and the cells alter in appearance. The protoplasm no longer fills the whole of the cell, and it becomes less clear. Large vacuoles appear, and many granules apparently chiefly of a fatty nature are to be seen. The shape of the cell also in many cases undergoes a change, becoming very irregular owing to processes developing at one or more points. (See fig. 2.) Such irregular cells, when placed in fresh beer-wort or beer-wort gelatine, produce buds in a typical yeast-like manner, and these buds grow out into the ordinary form of cell found in young colonies. (See fig. 3.) Besides these irregularly shaped cells there are also to be found in old colonies many spore-bearing cells. The morphology and nature of these will be described later.

The history of the manner of growth and the nature of the vegetation of the colonies is clearly shown by observing their production from single cells in cultures of hanging-drops of beer-wort gelatine at the ordinary room temperature.

Streak-cultures.

On the sloped surface of tubes of beer-wort gelatine at the ordinary room temperature a milky brownish-white streak is produced with well-marked and regular crenate edges. The vegetation of this streak resembles that of the colonies produced on plates of beer-wort gelatine.

On beer-wort agar (2 per cent. agar) the characters of the streak and of its vege-

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tation are similar. Streak-cultures on bread and potato are milky white when moist, and chalky looking when dry; on pieces of moist ginger their colour is darker, owing to the brown colour of the ginger. The cells are of the same form as in the other cultures on solid media.

A yeast ring is formed in old cultures on many nutrient liquid media, but no films are produced. In tubes of beer-wort the ring makes its appearance in 10–14 days at 25° C. It is milky white in colour, and looks like a layer of cream, deposited around the edges of the liquid. It is also formed at the ordinary room temperature, but more slowly. Its vegetation consists of yeast-like cells—oval, round, and a few elongated, sausage-shaped, irregular cells, like those in old cultures on beer-wort gelatine, and of cells containing spores.

Such rings are also formed on dextrose-Mayer, lævulose-Mayer, maltose-Mayer, and saccharose-Mayer solutions.*

Reproduction.

The methods of reproduction of this organism which have been observed are—(*a*), vegetative propagation by budding; (*b*), reproduction by the formation of spores.

(*a.*) *Budding.*—Budding takes place in a typical yeast-like manner, whenever cells of this organism are placed in suitable nutrient media. Beer-wort seems to be the best medium, and has been used both in its ordinary liquid state and made up into a solid medium with gelatine or agar, 10 per cent. of gelatine being used in the former case and 2 per cent. of agar in the latter. The rapidity of budding of course depends largely on external conditions, but an idea of the rate of growth of a vigorous cell was obtained by culture for 24 hours at 27° C. in beer-wort, and then making a hanging-drop culture of beer-wort gelatine of the vigorous cells so obtained, and observing the number of cells produced from a single cell in a given time at 15–19° C. The number of cells produced from a single vigorous cell kept at 15–19° C. was fourteen in 31 hours, but 21 hours were occupied in the formation of the first four cells. The temperature limits for budding show that this yeast is a distinctly high temperature form. Slow growth has been obtained with streak-cultures on beer-wort agar at 37–38° C.: above which temperature, however, growth seems to be practically stopped. The lower limit of growth appears to be between 10° C. and 13° C. Growth is most vigorous at 25–30° C.

(*b.*) *Spore-formation.*—Cells containing spores are to be found in all old cultures of this organism on the various media in which beer-wort has been used as a constituent. They are also found in the growths on moist bread, potato, ginger, and other nutrient media. The methods adopted for obtaining a plentiful supply of spores were the same as those generally used for obtaining spores of the various species of Saccharo-

* *I.e.*, MAYER'S solution made up with the corresponding sugar.

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myces, viz., a vigorous culture was obtained by growing the cells in beer-wort at 25° C. for 24 hours, then infecting a second supply of beer-wort and keeping this at the same temperature for the same length of time. A few drops of this culture were then poured on to a sterile block of gypsum or a piece of porous porcelain, which was kept in a moist chamber at 25° C. In the course of two days an abundant supply of cells containing spores was to be found on the porous surface. The spore-containing cells are entirely different from those of the ordinary species of Saccharomyces. They consist of two cells joined together by a neck, the cavities of the two cells being continuous through the neck. At first sight it appears as though an ordinary yeast-like cell had produced a bud, which, instead of being budded off in the usual way, had remained attached to the mother-cells by a wide neck, communication between the two cells not having been cut off at that point. The neck in most cases would then appear to have undergone a development in length and sometimes also in breadth. The number of spores in such a structure or compound cell is variable, and their arrangement also. Four seems to be the typical number of the spores, two in each compartment of the compound cell. They may, however, be arranged three in one compartment and one in the other, especially if one compartment be larger than the other; or, again, four in one compartment and none in the other, in which case the compartment without spores is usually very small indeed, and may appear as a mere process of the other. In a great number of cases, however, three or two, and, more rarely, five spores are found in these compound cells. In cases where three spores are present, two are in one compartment and one in the other, or occasionally three in the one and none in the other. If two spores only are present, one in each compartment seems to be the usual arrangement; if five, three may be in one compartment and two in the other, or four in the one and one in the other (fig. 4). The spores themselves are round or, more rarely, oval in shape; their diameter is 4-5 μ. They are strongly refractive in appearance and usually fill up the greater portion of the cavity of the compound cell, the cell-walls of which are kept in such cases on the stretch and retain their original ovoid form. In those cases, however, in which the spores do not occupy the major portion of the cell's interior, the walls shrink or undergo a certain amount of folding, and the outline of the compound cell then becomes irregular; and in these cases the resemblance to a pair of yeast-cells, joined by their necks, often becomes lost.

The various stages of development of the compound cells and of the spores have been observed in hanging drops of sterile distilled water, in which have been placed a considerable number of vigorously growing cells, obtained in the same manner as those used for the gypsum-block spore cultures. The hanging drops were kept under observation under the microscope at a temperature of about 25° C. The first indications of change taking place in the cells were the gradual loss of their homogeneous appearance and the development of bright-looking granules. At the same time many of the cells produced one or two small buds. In 12–48 hours after sowing it was

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noticed that several cells were producing small outgrowths, which in the earliest stages could not be distinguished from very young buds. As these projections grew larger, however, it was seen that they were not young forms of buds, but outgrowths of a different nature. They grew in length without becoming swollen as is the case with buds, and without any noticeable increase in width, until they presented the appearance of small beak-like tubular processes.

Having selected for special observation two cells close together and each with a single protuberance of the kind just described, the tips of the beaks pointing towards one another, the further behaviour of these structures was discovered (fig. 5, *a*). The small tubes grew in length until their tips touched (fig. 5, *b*). A gradual fusion then took place at the meeting point, the walls being apparently dissolved at that spot (fig. 5, *c–f*). The disappearance of the transverse walls was followed by the fusion of the protoplasmic contents of the tubes, so that the protoplasm in either of the cells was made continuous with that in the other by the connecting band running through the fused tubes. The walls at the point of contact also became continuous, possibly by the formation of cementing cellulose by the protoplasm at that point (fig. 5, *f–h*).

The appearance of the compound cells, thus formed, was that of two ordinary cells attached to one another by an elongated neck with complete communication of their cavities through the neck, and, at this stage, with protoplasm filling up the whole of the cells and the neck. A few hours after fusion in each compartment of the compound cell the protoplasm began to contract, and the bright granules, mentioned above, arranged themselves more or less into two groups. The contracted protoplasmic mass soon after showed signs of division into two rounded masses, and later on all signs of a single mass was lost, and the outlines of the two bodies were well marked. In connection with each of these was to be found one of the groups of bright granules (fig. 5, *h–k*). The rounded bodies of protoplasm turned out to be the young stages of spores. They developed into ripe spores by continuing the rounding-off process, until each appeared as a well-defined spherical mass, and by completing it by the deposition of cell-walls around themselves (fig. 5, *k–n*). In the case described, two spores were formed in each half of the compound cell, but, as has been already stated, their number varies in different cases.

The formation of spores in this manner took place more slowly and with less certainty in hanging-drop cultures of distilled water than on porous gypsum plates. This is no doubt due, in part at any rate, to deficient aëration in the former case as compared with the latter. To obviate this to some extent, cultures for spores were made on gypsum plates, as described above, and were allowed to remain at 25° C. until examination of the vegetation showed the existence of cells which were just beginning to put out tubes for conjugation. Some of these cells were then transferred to a hanging drop of distilled water and the subsequent stages observed as before. For a few hours the further development of the tubes seemed to be

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inhibited, probably owing to the sudden change of conditions, but afterwards they grew in normal fashion, at any rate in some cases.

In connection with the culture of this yeast in hanging drops of distilled water for the observation of the different stages in the process of spore-formation, use was made of WARD'S cells and water drops, as well as drops of dilute glycerine, since in experiments on the growth of the yeast in different media it had been found that if an infected drop of dilute glycerine was placed on a sterile glass slip in a moist chamber at 25° C., many conjugated cells with or without spores, and cells with tubes in various stages of development, were produced.

In other cases ring-cells were used, and after a few trials satisfactory results were obtained by placing a single drop of distilled water or of dilute glycerine on the slide at the base of the cell, and then sealing on the cover-slip, on the underside of which was the infected drop of distilled water. In this manner conjugation was obtained in a large majority of the drops. When drops of dilute glycerine, of strengths varying from 1 per cent. to 25 per cent. of pure glycerine, were used instead of drops of distilled water, conjugation took place as freely as in those cases where water alone was used.

The size of the drop seems to be of considerable importance. When it consists of a mere film of liquid, an abundant supply of spores is usually obtained, but when it is large, budding seems to supersede spore-formation to a considerable extent, this behaviour doubtless depending largely on the nature of the aëration of the cells. The influence of this factor is shown further if a tube containing about 10 cub. centims. of distilled water is infected with vigorous cells.

In such a tube of distilled water, kept at 25° C., the production of spores is very small, and large numbers of very irregular cells are produced (fig. 6). Cells of this type show great variety in shape, and have already been mentioned as occurring in old colonies on plates of beer-wort gelatine and in hanging drops of the same medium. They may now be considered in greater detail, since their nature and form seem to receive explanation from facts observed in connection with spore-formation.

The irregular cells are derived from ordinary yeast-like cells. The latter put out small projections or processes, which, at first, cannot be distinguished from very young buds, but which subsequently resemble the beaks put out by the cells in the process of spore-formation. There may be as many as three or four such processes developed from a single cell, in which case its resemblance to a yeast-cell is very small. These processes again may put out small protuberances, and in cases where these develop to any pronounced extent, a branching structure is produced. Other processes may swell up to a greater or less extent, in which case they resemble daughter-cells attached to the mother-cell, with communication between them. A small oval or round cell is often completely budded off at the tip of such a process, remaining attached to it at the point of origin of the bud. In other cases, instead

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of tube-like protuberances the processes are short and blunt, like the pseudopodia of an amœboid cell (see fig. 2). These are especially abundant in old cultures on beer-wort gelatine. If spore-cultures are made in the manner described for such cultures on gypsum blocks, but using sterilised pieces of an ordinary plant pot or of other less porous substances in place of the gypsum blocks, the development of irregular cells is very striking. Most of the cells in such a culture show more or less irregularity after 48 hours, and the irregularity in most cases is even more pronounced than that of the cells in the cases mentioned above. In some cells with a single protuberance the latter is so well developed, that the whole might almost be taken for a germinating spore (see fig. 7). Cells containing spores are to be found, but they occur with much less frequency than in cultures on plates of more porous material. Moreover, their shapes in a large number of cases are much more varied and irregular than those of the spore-containing cells developed on gypsum plates, or in hanging drops of distilled water, and the same feature is noticeable in the compound cells formed in distilled water. Many compound cells of the greatest irregularity of form are also found, in which no spores develop.

Vigorous cells were placed in a solution of MAYER'S solution* to which 2 per cent. of peptone had been added, and this culture was kept at 25° C. In 3–4 weeks, irregular cells were found in abundance and were more complicated than those produced by any other method used, compound cells of the most varied shapes being seen, but comparatively few contained spores (see fig. 8). There seems to be little doubt that these irregular cells, in most cases at any rate, are the results of attempts on the part of the ordinary yeast-like cells at spore-formation. The tube-like processes are, when unbranched and not swollen, exactly similar in appearance to, although longer than, the tubes which have been observed in hanging drops of distilled water, and which play an active part in the conjugation which precedes spore-formation. The greater length of the former is explained by a consideration of the conditions under which the tubes are developed in the two cases. It has already been stated that the best development of spores in hanging drops of distilled water has been attained when the drop consists of a mere film of liquid. The cells in this film, which take part in the spore-formation, are found very close together, and the development of a tube by any cell seems in most cases to be answered by the development of a similar tube towards it from the cell nearest to it. In those cases, where no such corresponding tube is produced, it is perhaps allowable to suppose that the cell is not in the right stage or condition to respond. The more nearly the conditions approach those which have been shown, by HANSEN (1) and KLEBS (5),

* Ammonium tartrate . . . . . 1 gramme.
Potassium phosphate . . . . . ·5 „
Magnesium sulphate . . . . . ·25 „
Calcium phosphate . . . . . ·05 „
Distilled water. . . . . 100 cub. centims.

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to be conducive to abundant and successful spore-formation, the better the response we may expect to find on the part of such a neighbouring cell. This may explain why there is a relatively abundant spore-formation in cases where the drop is nothing more than a film of water, and also in gypsum plate-cultures, and also why the tubes are comparatively short. For in these cases the conditions are as favourable as possible, and consequently the chances that any two neighbouring cells will combine together in spore-formation are relatively great. But in other cases where the conditions are not so favourable—*e.g.*, in tubes of distilled water, large hanging drops of water, and spore-cultures on plates of a poorly porous material, where the aëration is not so good as in the previous cases; or in old cultures on solid media, such as beer-wort gelatine, where the cells themselves are often not sufficiently vigorous; and in old cultures in liquid media, such as peptone Mayer solution, where not only the cells are not sufficiently vigorous for the most part but also the aëration is deficient—the chances that two neighbouring cells will unite in spore-formation are relatively small, and when a union takes place, the neck, composed of the two fused tubes, will generally be relatively long, for the cells capable of taking part in such a union are not so likely to be found so near together as in those cases where all conditions are favourable. At the same time cells which have to provide long tubes before conjugation can take place, must suffer a considerable loss of energy in the production of the tubes, and the surrounding conditions must make it very difficult to replace that loss. In consequence of this one would expect to find many cases in which the further development of the tubes had ceased after a certain amount of growth, and also cases in which fusion of the tubes had occurred, but no spore-formation had taken place on account of exhaustion. The observed facts suggest this view.

These considerations, then, explain both the increased length of the tubes and also the decreased spore-formation of cells under the less favourable conditions, that have been noted. They help to explain, moreover, the irregularities of the cells under such conditions. It seems in the highest degree probable that there is some kind of stimulus, chemical or otherwise, which acts on the cells which take part in spore-formation, both preparatory to and during the course of the growth of the tubes. Without supposing the existence of a stimulus, it is difficult to account for the answering development and the direction of the course of any pair of tubes. Under favourable conditions this stimulus acts between a pair of cells, situated very near to one another and capable of showing ready response to its action. Such a pair of cells may be considered to be dominated by this stimulus on account of their proximity and of the vigour of their response to it, and for these reasons they may be looked upon as indifferent to the action of similar stimuli, coming from other directions. But when the cases of cells under less favourable conditions are taken into account, it will be seen that such cases as that just described may be comparatively rare. Most of such cells are no longer practically dominated by one stimulus.

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Those capable of responding to the stimulus are more widely separated and consequently may be influenced simultaneously by stimuli coming from different directions, and may show a response to each of these by producing tubes in various directions. The development of these tubes may continue until the cell is exhausted, or until conjugation takes place in one case, or until, for some reason, the stimulus from one direction exerts a greater influence than the rest, and growth is more or less concentrated in that direction. Or again, a cell may respond to a stimulus in one direction only, and after a certain amount of development has taken place, the stimulus might cease to act (as one would expect frequently to happen where conditions are not very favourable): a stimulus in a different direction may then influence the cell, and call forth the development of a tube in the new direction. In such and similar ways cells of the most irregular shapes could be produced. Two or more cells may answer to the same stimulus, which will explain the existence of cell-groups in which two of the cells have conjugated in a normal manner, while at the point of fusion is attached the tip of a tube coming from a third cell; or the similar attachment of such a tube to a second tube from one of the conjugated cells may occur (see fig. 9). The tendency of tubes, which have no opportunity of conjugating, to swell up into bud-like forms or blunt processes has already been noted. The irregular cells, then, may fairly be considered as derived from ordinary yeast-like cells, which, under the various influences mentioned, have made attempts to produce spores by conjugation, and in consequence have undergone in many cases a complete change in form.

The Behaviour of the Nuclear Apparatus in Conjugation.

On account of the unique character of the method of spore-formation, a complete account of the behaviour of the nucleus, or its equivalent, would be very interesting. Unfortunately the nature of the nucleus in a yeast-cell is still a matter of controversy, and it would be outside the scope of this paper to enter into a discussion on that subject. Nevertheless, certain interesting and suggestive appearances were discovered when the cells of this organism in different stages of conjugation were hardened and stained.

The method adopted was as follows:—

Spore-cultures on pieces of porous porcelain plate were made and placed at 25° C. for a length of time, varying in different cases from 16 to 30 hours, so as to insure the occurrence of cells at all different stages of conjugation. The pieces of porcelain were then placed bodily in the hardening fluid, and, after hardening, cells were scraped off from the surface of the porcelain and placed in a drop of water on a glass slide. They were thoroughly mixed in this drop so as to separate the individual cells, and the drop was then allowed to evaporate, giving a layer of cells fixed on the slide. These were then stained on the slide.

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Various hardening fluids were tried, viz., a solution of iodine in potassium iodide solution, MERKEL'S solution, and RATH'S solution.

Among the different stains used were fuchsin-methyl green, as used by WAGER (2); the hæmatoxylin-iron alum method, as used by HOFFMEISTER (3); eosin and toluidine blue, as described by Miss HUIE (4); and the Flemming triple stain (11).

Separate cells, similar in appearance to an ordinary ovoid yeast-cell, showed in most cases a round, darkly stained mass in the centre of the cell, and situated around or in connection with this was a less deeply stained portion, which in well-stained preparations seemed to be made up of numerous deeply stained granules. The granular structure, however, was not by any means always to be seen. The rest of the cell was only slightly stained, or (with hæmatoxylin especially) not at all. It contained, however, well-stained granules, in groups or separate, distributed in the non-staining portion (fig. 10, *a*).

Cells with a small tube just beginning to be developed, showed in most cases the deeply stained mass situated at the point of origin of the tube, while a portion of it filled up the tube (fig. 10, *b*). The same appearance was observed when the tube had attained a greater length; but in cases where the tube was very long, the mass did not usually fill the whole of the tube, and sometimes did not project into it at all. Since, however, as a rule, fusion takes place, under the conditions of the experiment from which these preparations were made, when the tubes are quite short or of only a moderate length, this appearance can be taken as denoting an abnormal stage.

Cells which, as far as can be judged from their appearance, have just conjugated, showed a darkly staining mass filling the whole of the neck connecting them with a portion projecting at each end into the cells (fig. 10, *c*). Both in such cases and in those of cells before conjugation there was an irregular network of deeply stained granules distributed throughout the cell.

Thus far what happens in conjugation seems clear. The deeply stained central mass in the ovoid yeast-like cell undoubtedly represents a part at least of the nuclear apparatus. In some cases, where it is particularly well defined and stained, it is impossible to distinguish any difference between it and the nucleus of an ordinary cell; but in some cells no trace of such a body can be found, unless it is represented by some of the deeply staining granules distributed throughout the cell. However, this deeply stained body shifts its position from the centre of the cell to the point of origin of the conjugating tube, when this begins to appear, and as this develops protrudes into it, remaining there until conjugation, and then fusing with the similar mass in the tube of the other cell taking part in the conjugation. Thus the process of conjugation seems to be accompanied by nuclear fusion, and it is for this reason probably a sexual process. Whether this fusion merely represents the fusion of two specialised portions of the nuclear apparatus, or whether the darkly stained portions at the moment of fusion represent the whole of the nuclear apparatus of the

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conjugating cells, can only be determined when the exact nature of that apparatus is properly understood.

What happens after this stage is not so clear. Some compound cells showed a deeply stained mass lying at the mouth of the connecting tube in one cell with a portion running a little way across into the other cell (fig. 10, *d*). The latter showed no deeply stained mass, but contained an irregular network of granules (presumably the cytoplasm), and a similar network was also to be seen in the former cell. Fig. 10, *c*, and fig. 10, *d*, may be objected to on the ground of their resemblance to an ordinary yeast-cell with a bud: they were selected on account of their wide connecting necks, thereby showing with especial clearness the exact position of the deeply stained mass. They represent, however, two stages which are quite commonly found in compound cells, about the true nature of which there can be no doubt.

Other compound cells showed the deeply stained mass contracted entirely into one cell, the neck and the other cell being devoid of such a mass (fig. 10, *e*). Other instances were observed in which a more or less irregular mass was found in each compartment of a compound cell both in the neighbourhood of the neck, sometimes one or both extending into the neck, and around these masses was grouped, more or less completely, the granular network, which was very prominent and deeply stained (fig. 10, *f*).

Later stages of the same were also seen, but in these the deeply stained masses in each compartment, or sometimes in one only, showed signs of rounding off into spherical bodies (fig. 10, *g*), and still later stages showed that these were the rudimentary spores (fig. 10, *h*). Around these the granular network was arranged, becoming separated into two groups as the spores became better defined, one group around each spore. In older cells ripe spores were found, with lightly stained walls and deeply stained contents (fig. 10, *i*). Whether a definite karyokinesis occurs in the process could not be determined.

The history of the deeply stained body seems to be clear from the stage where two such bodies are found in the compound cell, one in each compartment. What takes place in such a case (assuming that four spores, two in each compartment, are finally formed, as is usually the case) seems to be the division of each body into two, accompanied by an aggregation and subsequent division of granules into two groups, one going to each spore, and each of the masses so produced takes part in the formation, and serves as the foundation of a spore. The earlier history of the fused deeply stained body is not obvious. There appears to be a contraction of the fused mass into one compartment, and, if this be the case, there must be a subsequent division of this and the return of one-half into the other compartment. Whether this division takes place in the former compartment, or whether a portion of the mass projects across the neck into the other cell and then division into two takes place, is not known; for in certain cases either interpretation may fairly be given to the appearances seen.

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The size of the deeply stained masses in the cells varies considerably. Sometimes they are very small and inconspicuous, and comparatively lightly stained, while on other occasions they are large and at once catch the eye. The shape also seems to be variable. In the ordinary yeast-like ovoid cells they are usually round and well-defined; but in cells with tubes, and also after conjugation, they often appear to be irregular and not sharply defined.

The division of the mass immediately preceding spore-formation appears to be single. The mass elongates and becomes narrower in the middle and swollen at each end, so resembling an hour-glass. Eventually separation takes place across the middle. In many cases, however, the granular network is so prominent that these appearances are not seen, and a state resembling a kind of karyokinetic division is shown. The granular network seems to play a very active part in this stage of spore-formation, and it also may possibly be nuclear in nature.

The fusions which occur in connection with this process of spore-formation seem to be different in nature from the fusions of hyphæ which have been observed in many Fungi, owing to the nuclear behaviour and the formation of spores as a sequel to the fusion. Marshall Ward (6) has described the hyphal fusions occurring in a Botrytis form, parasitic on *Lilium candidum*, and the only feature in common between the two types of fusion seems to be the development of the two parts which eventually fuse, answering to some kind of stimulus acting between them. Brefeld (7) and others have described the sporidial fusions of the Ustilagineæ, which are probably similar in nature to the ordinary hyphal fusions. Kihlmann (8) also has described the fusions taking place in *Pyronema*. The conjugation of this yeast form finds its parallel, perhaps, in the sexual fusions, preceding ascospore formation, of such Ascomycetes as *Eremascus*. The nearest approach to it, however, is the fusion of cells preceding spore-formation of the yeast *Schizosaccharomyces octosporus*, as observed by Schiönning (9), the account of which I have taken from Jörgensen's text-book on 'Micro-organisms and Fermentation' (10). In this form a single yeast-like cell divides into two daughter-cells by the formation of a partition wall, and these separate until they only remain attached together at one point. They then again coalesce, and at last form a lengthened ellipsoidal, hour-glass shaped, or irregular cell, which gradually increases in bulk, and within which a varying number of spores (usually eight) are formed. Hoffmeister (3) supplements this account by a description of the nuclear behaviour. Using the hæmatoxylin iron-alum staining method, he found a darkly stained body, which he considers nuclear in nature, in each of the daughter-cells. Fusion of these daughter-cells was followed by fusion of the darkly stained bodies. The single "nucleus" so formed then divides into two, and again into four and eight (or a number corresponding to the number of spores eventually produced), and around each of these daughter-"nuclei" a mass of protoplasm aggregates, and from each such combination a ripe spore is developed. It will be noticed that this process of spore-formation and the one described above corre-

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spond in essential points, the only distinction being that the manner of conjugation differs. Further, it will be seen that this similarity affords a link for binding together the "budding" yeast-like organisms and the fission "yeasts."

Germination of the Spores.

The germination of the spores has been observed in hanging-drop cultures. A tube of beer-wort gelatine was infected with cells from a gypsum block spore-culture, many of which contained ripe spores. The tube was heated for 5 minutes at 55° C. in order to kill the ordinary vegetative cells, which were mixed with the spore-containing cells, and which might otherwise have interfered with the observation of the germinating spores, owing to their rapid budding. Hanging drops were then made from the contents of the tube, and compound cells in the drops were kept under observation at 18–19° C. About 24 hours after making the drops the spores began to swell, and when the diameter was nearly doubled, they began to bud like ordinary yeast-cells, and their development continued in this manner, a colony of ovoid and round yeast-like cells being ultimately produced (fig. 11): the fate of the mother-cell walls was not clearly seen in most cases, it being difficult to make out whether splitting or gradual solution took place. When, however, a tube of beer-wort was used instead of beer-wort gelatine, and kept at 25° C., examination of the contents after 24–48 hours showed many germinating spores in all stages, and a large number of these were still entangled in the mother-cell walls. In such cases it was seen that the mother-cell walls were ruptured by the swelling of the spores within, and that the latter made their escape by that means (fig. 12).

Temperature Limits for Spore-formation.

The spores are formed most easily and quickly between 25 and 30° C. Using the gypsum-block method, at 25–27° C., the first signs of spores (fig. 13) in the compound cells appear in 20–24 hours; and at 34° C. in 32–36 hours. Above and below these temperatures, spore-formation takes place more slowly. At 36–37° C. spores appear in 2–3 days, and none seem to be formed at a higher temperature than 37–38° C. At 13–15° C. 10–14 days are required, and below 13° C. practically no spores are formed. After 16 hours at 26° C. many cells with the small tube-like projections can be found, and in some cases conjugation has taken place (fig. 14). At 33° C. this process takes place rather more slowly.

The newly ripened spores are generally killed by heating in beer-wort for 10 minutes at 60° C., but in some cases they survive exposure to a temperature of 65° C. for 5 minutes.

Fermentation.

A fairly brisk fermentation occurs in tubes of beer-wort, especially if the wort be infected with vigorously growing cells which have been cultivated at 25° C. for

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24 hours. Fermentation begins to be noticeable in 10–24 hours after infection at 25° C. and continues for 2 or 3 days. If the fermented wort be distilled and the distillate subjected to the iodoform test, crystals of iodoform are formed, proving the presence of alcohol. The fermentation of beer-wort has been noticed in every case in which beer-wort has been used for cultivating the organism. Lævulose is also fermented vigorously, and dextrose and saccharose slightly. Maltose, lactose, and dextrin are not fermented. A mixture of dextrose with maltose and dextrin is fermented more freely than dextrose alone. With regard to the fermentations of dextrose and saccharine, while in many cases comparatively vigorous fermentations were obtained, there were nevertheless many cases in which no visible sign of fermentation was seen, although apparently the conditions were identical, and no outside infection, which might have caused the alcoholic fermentation, had occurred. Long-continued cultivation in beer-wort seems to have increased its fermentative activity for that medium.

General Conclusions.

In conclusion, there seem to be three possible views regarding the nature of this fusion-process or conjugation of yeast-cells, viz.: (1) it is a mere cell-fusion such as so frequently occurs between contiguous cells in fungi; (2) it is a true sexual process, such as is now known to occur in many fungi; or (3) it is an abnormal or pathological phenomenon due to the conditions of culture.

Taking the last possibility first. Its improbability seems demonstrated on reflecting that the result of the process is the production of normal healthy spores, capable of germinating, and that the conditions are exactly such as are generally efficacious in the production of spores in yeasts of all kinds. True, the process is one of starvation, so far as organic food-materials are concerned, but KLEBS has shown clearly that just such methods of suddenly starving previously well-nourished organisms usually result in the development of sexual organs. The process seems to consist in the cell suddenly being driven to utilise its stores, in presence of free oxygen, and at a suitable temperature, and one expression of the results is the accumulation of nuclear substance in the non-growing cell. Another is the reaction of the nucleated protoplasm on neighbouring masses; and the exertion of chemotropic attractions.

Passing now to the second of the three possibilities given above. It would appear that in this conjugation of yeast-cells we have the protrusion of tubular beak-like processes in two opposed cells, each of which has accumulated quantities of sporogenous nucleated protoplasm. The movements of the beaks each towards the other, and the peculiar curvatures often shown, suggest mutual attraction, of the kind apparent in a conjugating *Mucor* or *Spirogyra*, prior to the formation of zygo-spores. The presence of the nuclear substance in the young beak (fig. 10, *b*) suggests that it is actively concerned in exerting this attraction, or in forming the beak, or both; and its further behaviour in the conjugating process (fig. 10, *c*, and *d*) points to a mingling

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of the nuclear substance as in a true sexual process. These seem good grounds on which to base the conclusion that we have here a true—though very simple—sexual act, leading to the formation of spores in one or both of the conjugating cells.

Let us now examine the first possibility mentioned above. Is the case before us merely one of cell-fusions, such as frequently occur in the anastomoses between hyphæ? There are facts among the true yeasts to support such a possibility, for HANSEN found that the germinal tubes arising from spores of *Saccharomyces Ludwigii* (HANS.) often fuse along their course;* but it must not be overlooked that in the present case the cell-fusions occur not between *growing* young cells, but at the end of growth, at a time when much nuclear substance has been accumulated but the cells themselves are not actively growing. Moreover, although we are still in want of information as to details, it does not appear that in ordinary cell-fusions the nucleus, or nuclear substance, plays any essential part beyond—possibly—starting the softening of the cell-walls, and perhaps initiating the attractive processes which lead to the mutual putting forth of the fusing beaks. At any rate it is generally admitted that nuclear fusions do not occur in such cases, and since in the present instance the nuclear substance appears to mingle, and the fusions take place as a preliminary to spore-formation, and after the cessation of active growth, the process appears to be more important than mere anastomoses of hyphæ.

To the three possibilities mentioned and discussed above we might add yet another, viz., that the act of union might conceivably be one of parasitism, were it not that the similar and equal reciprocal action of each cell, and the certainty that the cultures are pure, preclude any such conclusion, unless we are prepared to extend it to other cases of sexual union.

In a certain sense it may be true that a sexual union may be an act of *autophagy*,† and the frequent cases of union between the nuclei of neighbouring cells, recently divided off from one another, often present suggestive resemblances to parasitism; but in the case before us the act of conjugation directly precedes the definite formation of spores, and thus seems to be an example of sexual union at least as clear as that of conjugation between zoospores.

There is one aspect of all simple sexual acts that appears worthy of further attention, and which is suggested very definitely by the present case. It would appear that whenever nucleated spore-producing protoplasm is being stored in neighbouring cells, or whenever it has been accumulated and is parted by a septum, a strong tendency exists for union. Numerous examples can be given, such as the fusions of sporidia of Ustilaginæ or of Protomyces, the nuclear fusions in Teleutospores, Æcidiospores, &c., or those in the ascogenous cells of Erysipheæ, *Exoascus*, *Peziza*, &c., and in the basidia of Hymenomycetes. Also the fusion of sister-cells in some Conjugatæ, *e.g.*, *Spirogyra*.

* Similarly with the imperfectly known *S. Comesii* (CAV.).

† DANGEARD, 'Programme d'un Essai sur la Reproduction sexuelle,' p. 3 of reprint.

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On the other hand, cell-fusions—whether accompanied by nuclear fusion or not—are common in cases were no such accumulation of sporogenous substance can be postulated, *e.g.*, the nuclear fusions in the embryo-sac, the formation of clamp-connections in the mycelium of Basidiomycetes and of anastomoses in the hyphæ of Ascomycetes, &c.

How far this may mean that unions which are not necessary to the species—and not truly sexual—may occur in fungi, cannot be determined so long as our only criterion of a sexual process is the mere fusion of nuclei. If in *Sphærotheca* nuclear fusion occurs between the oogonium and antheridium, and again occurs between the two nuclei in the sub-apical cell of the ascogenous filament, as HARPER asserts, we appear to be in a difficulty as to which is the sexual process. OLTMANNS seems to have solved the difficulty in the case of the—apparent—double process in Florideæ, by showing that while nuclear fusion occurs in the union between spermatium and oosphere, it is absent from the subsequent unions between the ooblastemata and auxiliary cells. But the difficulty again crops up in phanerogams, in which observers already speak of "double fertilisation"—one between the oosphere and the sperm-nucleus, and another between the definitive nucleus and the second sperm-nucleus; meanwhile there is yet to be explained the fusion of the two embryo-sac nuclei to form the definitive nucleus.

Do all these phenomena point to the fact that wherever nuclear substance can gain energy by fusion with nuclear substance of slight difference of potential, this is effected; and are we justified in concluding that whenever such a union results in the formation and setting aside of a definite reproductive cell—sexual spore, zygote, fertilised egg-cell—that particular union may be termed sexual?

It would seem very probable that such is the case, and that sexual unions are only a particular case of fusions which are always apt to occur if nuclear substance comes near other nuclear substance within the sphere in which mutual attraction can make itself potent.

Be this as it may, it seems difficult on any grounds to reject the conclusion that we have in this case of a conjugating yeast, an unexpected example of a sexual process of the simplest kind.

Finally the question arises as to the systematic position of the yeast here described. In that it "buds" (pullulates), induces alcoholic fermentation, and forms one to five (usually two) spores in the cells, its characters accord with those of Saccharomyces, but no known Saccharomyces has its spore-formation preceded by an act of conjugation. It seems necessary therefore to propose a new genus, and on the analogy of Schizo-saccharomyces—the name given by BEYERINCK to a form which first divides into two cells which then fuse again as the spores develop—it might perhaps be called Zygo-saccharomyces.

The above work has been carried on at the Cambridge University Botanical

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Laboratory by permission of Professor MARSHALL WARD, to whom I should like to take this opportunity of expressing my thanks for his constant help and advice during the whole course of the work.

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DESCRIPTION OF PLATE 46.

Fig. 1. Early stages in the development of a colony from a single cell by budding, as observed in a hanging drop of beer-wort gelatine. × 750.

a. April 23, 1900, 12.15 P.M., temperature 19° C.
b. ,, 23, 5 P.M., 19° C.
c. ,, 24, 9.30 A.M., 19° C.
d. ,, 24, 11.30 A.M., 15·5° C.
e. ,, 24, 2.35 P.M., 16·5° C.
f. ,, 24, 7.15 P.M., 16·5° C.

Fig. 2. Irregular cells from a three weeks' old colony in a hanging drop of beer-wort gelatine at ordinary room temperature (15–20° C.). × 500.

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Fig. 3. Young colonies of ovoid and round yeast-like cells, developing from irregular cells, similar to those shown in fig. 2, when placed in a hanging drop of fresh beer-wort gelatine, after 36 hours at 15–20° C. × 750.

Fig. 4. Spore-containing and irregular cells, found on a gypsum-block spore culture, 2 days old, at 25° C. × 750.

Fig. 5. Stages in the formation of spores by conjugation of two cells, from a hanging drop of distilled water, made on December 4, 1900, and kept at about 25° C. × 1000.

a. Dec. 6, 10.55 A.M., 25° C. *b.* Dec. 6, 11.25 A.M., 26° C.
c. ,, 6, 11.55 A.M., 27° C. *d.* ,, 6, 12.30 P.M., 27° C.
e. ,, 6, 1.15 P.M., 27° C. *f.* ,, 6, 2.15 P.M., 27° C.
g. ,, 6, 4.45 P.M., 27° C. *h.* ,, 6, 6.30 P.M., 25° C.
i. ,, 6, 9.45 P.M., 23° C. *j.* ,, 7, 12, 5 A.M., 25° C.
k. ,, 7, 1 A.M., 26° C. *l.* ,, 7, 2. A.M., 24° C.
m. ,, 7, 3 A.M., 25° C. *n.* ,, 7, 11.50 A.M., 25° C.

Fig. 6. Irregular cells from a tube of distilled water, 4 days old, at 25° C. × 750.

Fig. 7. Irregular cells found in cultures for spores on pieces of plant pot, 27 hours old, at 25° C. × 750.

Fig 8. Irregular cells from a tube of peptone-Mayer's solution, 3 weeks old, at 25° C. × 750.

Fig. 9. Irregular cells from a tube of distilled water, 4 days old, at 25° C. × 750.

Fig. 10. Cells fixed with RATH's solution and stained with the Flemming triple stain, from spore-cultures on gypsum blocks, 10–30 hours old, at 25° C.

a. Ovoid cell, showing central darkly stained round body and well-stained granules. × 1200.

b. Ovoid cell with small tube just developing, showing darkly stained body, situated at the point of origin of the tube and projecting into it, and granular network. × 1200.

c. Compound cell, formed by conjugation of two cells, with darkly stained mass, stretching across the connecting tube from one cell to the other, and granular network. × 1300.

d. Compound cell with darkly stained body, situated in one cell only, and projecting partly into the connecting tube, and granular network. × 1300.

e. Compound cell, showing darkly stained mass, situated in one cell only, close to the opening of the connecting tube, and granular network. × 1400.

f. Compound cell, each compartment containing an elongated darkly stained body with an aggregation of the prominently shown granular network around it. × 1400.

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g. Compound cell, showing the division of the darkly stained mass in each compartment by constriction and the arrangement of the conspicuous deeply stained granular network. × 1400.

h. Compound cell, showing the rounding off of the divided portions of the deeply stained masses into spores, with deeply stained granules on their surfaces. × 1400.

i. Compound cell, containing ripe spores, whose walls are but lightly stained. × 1400.

Fig. 11. Germination of spores contained in compound cells as observed in hanging drops of beer-wort gelatine at ordinary room temperature (18–19° C.). × 750.

a.	Sept. 25, 2.30 P.M.	*b*.	Sept. 28, 11 A.M.
c.	,, 28, 3.30 P.M.	*d*.	,, 25, 12 A.M.
e.	,, 26, 4 P.M.	*f*.	,, 27, 9.30 A.M.
g.	,, 27, 1 P.M.	*h*.	,, 27, 4.30 P.M.

Fig. 12. Germinating spores from a tube of beer-wort, showing remains of the split mother-cell walls entangled among the germinating spores. × 500.

Fig. 13. Conjugated cells and cells with small tubes from a gypsum block spore-culture, 19¼ hours old, at 25° C. × 750.

Fig. 14. Conjugated cells with rudimentary spores from the same culture 3 hours later. × 750.

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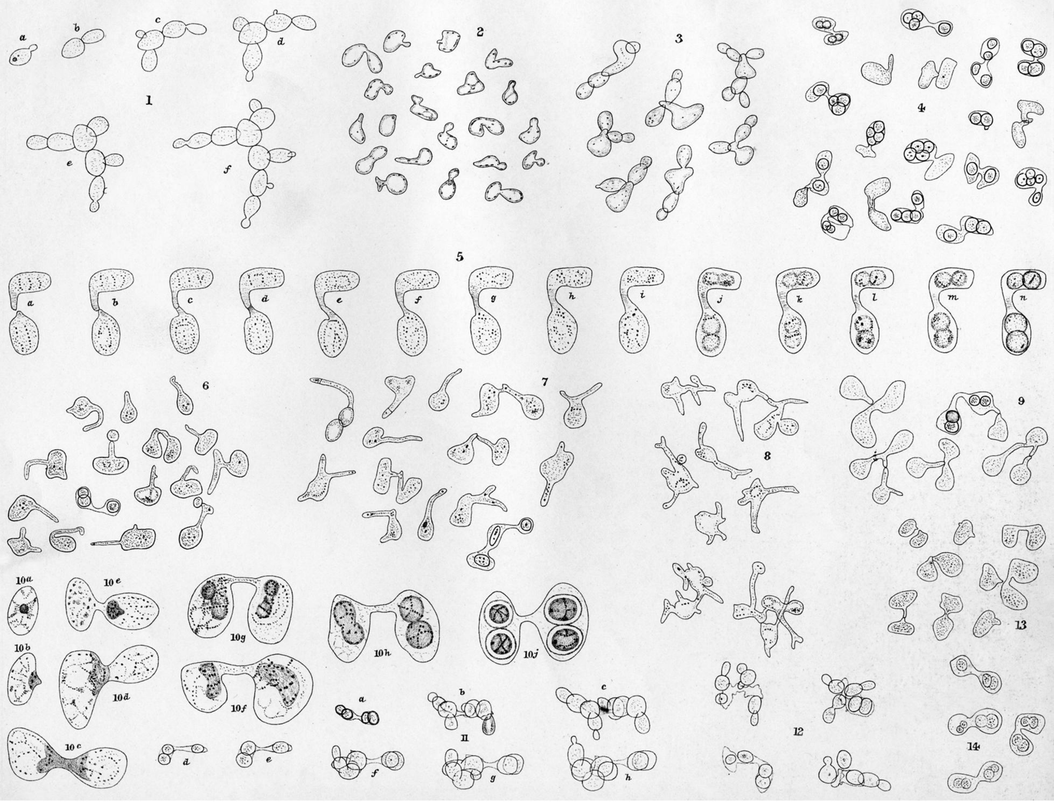

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